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An Experimental Study of Microbial Nest Associates of Borneo's Exploding Ants (*Camponotus* [*Colobopsis*] species)

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Abstract.—Cavity nesting ants in the *Camponotus* (*Colobopsis*) *cylindricus* (COCY) complex possess hugely hypertrophied mandibular gland (MG) reservoirs containing weakly acidic phenolic acetogenins and/or diterpenes unique for insects. Many taxa ("exploding ants") use these products in suicidal defense of territory, but major workers of all species, and all workers of some species, possess hypertrophied reservoirs and clade-typical products not used in suicidal fights. An additional role of MG products in nest hygiene was suspected. We sampled microbial associates of nest cavity fiber and carton shelving in artificial wooden nests occupied by substantial colony fragments of COCY species and compared them with two controls: microbes in unoccupied nests and nests occupied by other cavity-nesting ant species. Several natural nests in fallen wood were also sampled. Bacteria and fungi cultured on malt extract agar were identified from gene sequences amplified by universal bacterial and fungal primers. Results were related to an expanded data base on MG chemistry. Twenty-four of 55 nests were colonized by ants, mostly by COCY species, nesting naturally or not in dead wood. In colony-level analyses, mycoparasitic *Trichoderma* fungi were significantly over-represented in nest fiber of COCY species. Their detection was restricted to taxa naturally inhabiting fallen wood; the majority of these taxa produced *m*-cresol as the major component of MG volatiles. *Burkholderia* bacteria were significantly more common in COCY species' nests than in unoccupied nests but only when replicate nests per colony were allowed. *Trichoderma* and *Burkholderia* tended to co-occur in nest fiber, perhaps due to traits influencing arrival and survival. Both *Trichoderma* and *Burkholderia* may contribute to nest hygiene, and their joint occurrence could potentially affect longevity of nests in dead wood. Both genera also occur as endophytes, and interactions between ants and endophytes merit further study. Documented over-representation in live hosts of genera *Antidesma* and *Cleistanthus* [Phyllanthaceae]) could be related to the microbial environment provided by these hosts.

"From the information available, ants universally reject fungi ... as inquilines in their living quarters, although this generalization merits further investigation" (Sánchez-Peña 2005)

Eusociality has long been recognized as a life style conveying high vulnerability to pathogens (e.g. Hamilton 1972; Shykoff and Schmid-Hempel 1991). Extranidal activities regularly expose foragers to diverse microbes, including potential pathogens that may spread rapidly among numerous closely interacting and genetically similar individuals at the nest. Such threats are evident from the ants' early evolution of metapleural glands, located on the posterolateral mesosoma and functioning in

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antisepsis (e.g. Maschwitz et al. 1970; Maschwitz 1974; Macintosh et al. 1995; Bot et al. 2002; reviewed in Hölldobler and Wilson 1990). Unique to the ants, these exocrine glands produce mainly proteinaceous compounds (do Nascimento et al. 1996), augmented in at least some taxa by volatile organic components (do Nascimento et al. 1996; Ortius-Lechner et al. 2000; Jones et al. 2005).

Adaptations against detrimental nest microbes are best studied in leaf-cutter ants (tribe Attini), where diverse ant traits oppose both potential pathogens and a dangerous parasite of the fungal garden that sustains developing larvae (reviewed in Currie and Stewart 2001; Bot et al. 2001a; Hughes et al. 2002; Mueller et al. 2005; Fernández-Marín et al. 2006; Little et al. 2006; Zhang et al. 2007). Various metapleural gland volatiles are differentially effective against different categories and life history stages of microorganisms, and this diversity of compounds may guard against the evolution of resistance in pests (Bot et al. 2002). Complementing these secretions are specific, evolved behaviors including grooming, weeding, and waste management (reviewed in Bot et al. 2001a; Currie and Stewart 2001; Hart and Ratnieks 2001; Mueller et al. 2005; Fernández-Marín et al. 2006; Little et al. 2006; Zhang et al. 2007). Additionally, to oppose a dangerous parasite of the fungal garden (a fungus resistant to metapleural gland volatiles; Bot et al. 2002), workers maintain an antibiotic-producing natural enemy of the parasite (Currie et al. 1999a; Currie 2001; Gerardo et al. 2006; Little and Currie 2007, 2008). The beneficial actinomycete bacteria can potentially evolve rapidly to combat a constantly evolving pest (Currie et al. 1999b, 2003a,b; Currie 2001; Mueller et al. 2005; Poulsen et al. 2005; Little et al. 2006; but see Gerardo and Caldera 2007). Given the advantage of this strategy, it would be surprising if other ant taxa had not evolved to use beneficial microbes as antagonists to microbial enemies.

Despite near ubiquity of metapleural glands in the worker caste of ants, many members of the highly species-rich and cosmopolitan tribe Camponotini have lost the glands secondarily (Hölldobler and Engel-Siegel 1984). How might nest hygiene be maintained in these taxa? Possibilities include restriction of nests to less pathogen-plagued substrates, frequent movement of colonies to new nests, and/or transfer of antiseptic function to other glands (Maschwitz et al. 1970; Cole 1975). Alternatively, or in addition, these ants might exploit antiseptic properties of beneficial microbes. Further, because costs of anti-pathogen defense can be significant (e.g. Poulsen et al. 2002, 2003; Currie et al. 2003b), defensive costs might be reduced by basing defense mechanisms on nutrients present in abundance or excess. For example, in frequently nitrogen-limited camponotines (Davidson 2005), costs might be reduced by deploying defenses based on investments of carbon, rather than nitrogen, i.e., on volatile organics, rather than proteins.

To better understand resistance to nest pathogens in taxa lacking metapleural glands, we focused on a well-resolved 15-member clade of cavity-nesting camponotines in which variation in nesting habits likely correlates with differential exposure to nest pathogens (Cook 2008). Coexisting locally in a Bornean rain forest, species in the *Camponotus* (*Colobopsis*) *cylindricus* clade (hereafter COCY species) lack metapleural glands. (The informal subgenus *Colobopsis* appears to be a heterogeneous group, and this classification could change.) However, in most of these taxa, mandibular gland (MG) reservoirs have hypertrophied through the abdominal tip to fill much of the body cavity. Their products include phenolic acetogenins and/or diterpenes, as well as sugars that convey adhesive properties to the secretions (Jones et al. 2004). All of these components are nitrogen-free. Some of the phenolic acetogenins, i.e., the corro-

sively irritant m-cresol and resorcinol, possess known antiseptic activity, and others should be at least weakly antiseptic by virtue of their weak acidity. All COCY taxa forage by 'grazing' microscopic foods from adaxial leaf surfaces, mainly in the high canopy (Davidson et al. 2004), and they nest both polydomously, and wholly or partly within cavities of live trees. Canopy nesting is basal in the group, and a more derived trait is nesting low (0–3 m) in live trunks only. The most derived nesting habit includes both central nests in live trunks and satellite nests in dead wood. In four of five members of this last group, m-cresol is a prominent component of MG product. We expect that nest cavities in the arid canopy should be less pathogen-plagued than those in the wet understory, and that nests in fallen wood on the damp forest floor should pose the greatest threat from pathogens by offering conditions conducive to their growth. Densities of wood, and of root- and butt-rot cavities, should also be greater in the understory than in the canopy, and an ability to nest in dead wood should provide the greatest density of potential nest sites. To the extent that nesting space is limited, such limitations could have driven evolution of the capacity for increased use of pathogen-plagued nests.

Given the observed phylogenetic trend in COCY species' nesting habits, known antiseptic properties of MG compounds, and the desirability of defining mechanisms contributing to nest hygiene, we decided to assess microbial nest associates directly by comparing their presence in COCY-occupied versus unoccupied artificial nests. Opportunistically, we also sampled artificial nests colonized by non-COCY species and a few natural COCY nests in decaying wood. Cultured microbes from surface-sterilized nest wall fiber and carton shelving were preserved and identified by molecular sequencing using oligonucleotides targeting bacteria and fungi. In focusing on the subset of microbes culturable under a particular set of conditions and detectable with universal bacterial and fungal primers, we could have missed some regular microbial associates of the ants. However, our methods were chosen as a simple first approach to probing for regular relationships between ants and microbes in nesting environments where microbial diversity could be high. Studied in this experimental context, consistent over-representation of particular microbes within occupied nests can be related to ant nesting habits, to glandular chemistry (reported here for an expanded set of COCY species), and to known characteristics of the microbes in question.

Two other sets of observations complement the study of nest microbes. First, after opening both occupied and unoccupied nests for microbial sampling (authors' unpubl. data), we measured cavity wall pH. Motivating this measurement was variation in pH-dependent colors of ant MG products (Jones et al. 2004), and the observation that workers of one species applied MG products to plastic nest tubs in the laboratory. Nest wall pH was hypothesized to vary in relation to product color, and to potentially influence microbial affiliations with nest walls. Second, to the extent that ant occupancy of nests in live host trees may depend on establishment of appropriate microbial environments, we suspected non-random use of host trees. We therefore compared frequencies of host use against representation of plant families and genera in the data base of KBFSC tree plots. Working in a protected area, we could not follow these studies with destructive sampling of live hosts trees for microbes.

MATERIALS AND METHODS

Chemistry of Mandibular Gland Products

For taxa whose MG chemistry had not previously been studied, whole worker

ants were collected from individual colonies into approximately 0.5–1.0 ml of methanol and returned to laboratories at the Universiti of Brunei Darussalam (UBD) and the Virginia Military Institute (VMI) for analysis of supernatant by gas chromatography/mass spectrometry (GC/MS) Analytical methods were identical to those in an earlier publication reporting volatile chemistry of nine species (included here from Jones et al. 2004). Peaks were identified by comparison with coinjected compounds from commercial sources or synthesized by T. H. Jones at VMI.

Nest Construction and Sampling

In November-December 2005, two investigators (CRB and DWD) constructed 55 compositionally identical nests, and transported them in the field over five successive days in early December. Each nest consisted of a 2″ × 2″ piece of medium density dipterocarp lumber, approximately 42 cm long and sawn initially into three segments. With a power drill and a stout bit, we drilled two adjacent holes completely through the middle segment (from both ends and meeting in the center) and again, most of the way through both upper and lower segments. The same drill bit was used to eliminate partitions between adjacent holes. The three nest segments were tightly reassembled using wood glue and staples, and a hammer and nail (subsequently removed) were employed to make a single entrance hole near the top of the cavity in the upper nest segment. At points below and above the nest cavity space, intact nests were nailed to 1-m-tall stakes for insertion into the ground. Before nailing, both nests and stakes were given two coats of green, oil base paint, and nests were numbered with permanent marker. Numbered nests were matched haphazardly to colonies of different ant species and were placed either immediately adjacent to natural nests (N = 25) or a short distance (5–7 m) away, but connected by ropes along which workers readily commuted (N = 30). Four nests lacked stakes; two of these were tied directly to tree trunks, and two others, to canopy branches accessible from a walkway. Nests were placed near colonies of all but two COCY spp. known from KBFSC, though sample sizes were uneven and depended on species abundance.

To test for differences in nest-wall pH, we first verified neutral pH of test strips (colorpHast, Merck KGaA, Darmstadt, Germany) in tap water (= stream water) and then held wet strips against the cavity wall until their colors had ceased to change (usually < 1.5 min).

After sampling three COCY-colonized nests with large colony fragments in March–April, 2006, we retrieved nests and sampled microbes of both occupied and unoccupied nests at intervals of 4–6 months: in November–December 2006, July 2007, and November–December 2007. (The few samples from this last period were mostly unusable, perhaps because lab alcohol had been diluted.) Nests appearing to house few ants in early censuses were left for subsequent sampling periods. Harvesting occurred at night, with ants inactive and sealed inside; nests were returned to the KBFSC laboratory for processing. One or two days after sampling, nest segments were disassembled on an isolated table. Live workers and brood were brushed, usually without exploding, into one or more plastic tubs, ringed along their internal lips with an aqueous suspension of poly(tetrafluoroethylene), and covered with lids punctured for aeration. Individual nests were fractured into their original three segments, and nest fiber was extracted from the upper portion of the lowest nest segment; if present, brood were found most dependably in this segment. Selection of fiber lining the nest cavity was otherwise haphazard, and that of carton (falling from nests as ants were extracted), completely haphazard. Given such minimal sampling within nests, we would only expect to see microbial taxa occurring regularly across

samples if those taxa were very common and widespread within as well as across nests, and such taxa could occasionally be missed. For the few natural dead wood nests sampled, microbial sampling was similar, except that sites for sampling of nest lining (fiber) and carton were chosen haphazardly from within brood chambers. Carton and fiber samples were preserved separately in haphazardly selected and subsequently labeled 50-mm centrifuge tubes, washed recently in dilute sodium hypochlorite and then rinsed with tap water and air-dried. For nests lacking carton (unoccupied nests, some nests housing non-COCY species, and COCY nests lacking brood), only fiber samples were available. Live ants were fed honey water until their return to the field with reassembled nests, and some nests were sampled again on successive field trips. On the second through fourth field trips, various colony fragments were retained for observation.

After extracting samples, we sterilized a plexiglass chamber ("sterile hood"), approximately 36 cm on a side, with a tightly-fitted door. Internal chamber walls were swabbed with Kimwipes© soaked in 10% sodium hypochlorite and then 95% ETOH, which quickly dried them. Using a sterile razor blade, we haphazardly cut tiny fragments of nest wall fiber or carton samples and placed them individually by nest number and sample type into 1.5-ml microcentrifuge tubes containing 10% sodium hypochlorite for surface sterilization. (Forceps used to handle samples were sterilized in the flame of an alcohol lamp.) Subsequent sample agitation for 2 min was followed by two sequential 2-min rinses (with agitation) in microfuge tubes filled almost to capacity with sterile, deionized water. After drying on sterile filter paper inside the chamber, samples were transferred to small (50-mm diameter) sterile plates of Malt Extract Agar (MEA) and plated three per plate and widely separated. Taped plates were transferred to a second and identical "sterile hood" covered externally in aluminum foil to exclude light. Plates were checked daily, and when microbial growth around individual plated samples almost met that from other fragments, cultured microbes were harvested using sterile forceps, and with underlying agar, into 95% ETOH. Samples within a plate usually grew visually similar cultures; if so, such samples were combined. If microbial cultures within a plate differed in appearance, these were preserved individually. All preserved samples were returned to Utah for DNA extraction (below).

Sampling of Leaves and Roots

As the study progressed, it became clear that certain prominent nest microbes had previously been reported as endophytes, so we also sampled and identified endophytes from accessible resource plants. Although all COCY species forage principally in the canopy, workers from certain colonies also regularly grazed leaves of a few understory plants, and one heavily used tree canopy was reachable from a canopy walkway. We observed workers of species 'YG' and 'SA' in the understory, and of 'LE' in the canopy, and circled (in permanent ink) 'leaf-stops' where foragers paused to graze adaxial leaf surfaces. Because the endophytic microorganisms in question can also be root parasitic, we sampled shallow roots of understory resource plants used consistently by colonies of 'BBQ', 'YG', and nrSA. Sampling of leaf and root tissues from COCY resource plants was exploratory and not sufficiently replicated to test hypotheses of ant association with particular endophytic microbes in the foraging territory. Whole leaves or bits of root tissue were harvested and bagged individually in new plastic zipper-sealed bags and returned to the KBFSC laboratory for surface-sterilization and culturing using techniques identical to those for nest fiber and carton.

DNA Extraction, PCR, Sequencing, and Microbial Identifications

DNA extraction was carried out using Qiagen's DNeasy® Tissue Kit (QIAGEN®, Valencia, CA), following manufacturer's specifications for the Purification of Total DNA from Animal Tissues (Spin-Column Protocol), though modifying step one. That step (tissue shredding and grinding), was carried out in a 1.5 ml microfuge tube after first freezing a portion of the sample in liquid nitrogen. PCR amplifications utilized universal primers for the bacterial 16S rRNA gene (27f, 5'-AGAGTTTGATCC TGGCTCAG and 1492r, 5'-GGTTACCTTG TTACGACTT) and the fungal 18S rRNA gene (nu-SSU-0817, 5'-TTAGCATGGAA-TAATRRAATAGGA and nu-SUU-1536, 5'-ATTGCAATGCYCTATCCCCA). A 2-µl DNA sample was added to a 42.3 µl reaction mixture consisting of 11.2 mM Tris-HCL (pH 8.8), 59 mM KCL, 0.38 mM dNTP mix, 1.5 mM MgCl2, 0.5 µg BSA, 0.38 µM of each primer, and 2 U Taq DNA Polymerase. PCRs were carried out on a MiniCycler™ PTC-150 (MJ Research Inc., Watertown, MA), with published protocols slightly altered to decrease false priming. The amplification protocol for bacterial primers typically consisted of a denaturing step of 95°C for 2 min, followed by 35 cycles of 90°C for 45 sec, 50°C for 45 sec, and 72°C for 1.5 min; it concluded with a final extension step at 72°C for 7 min. With fungal primers, the protocol typically consisted of a denaturing step of 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; it concluded with a final extension step at 72°C for 10 min. PCR products were purified using Qiagen's QIAquick®PCR Purification KIT, and following the manufacturer's protocol for use of a microcentrifuge.

PCR amplicons were sequenced directly using ABI dye-terminator chemistry at the DNA Core Facility, University of Utah School of Medicine. Forward and reverse sequences were assembled and edited using Sequencher v 4.5 (GeneCodes 2005). Related sequences were aligned in ClustalX (2.0) to check variable positions and make minor adjustments. Consensus sequences were entered into BLAST (National Center for Biotechnology Information-GenBank) for identification. We present mainly genus-level identifications; species level determinations are tentative due to occasional availability of just partial sequences (typical for environmental samples), and to limitations of the BLAST data base for gene regions studied.

Ant-host Associations

During two research trips, we cut, pressed, and dried vegetative material from accessible live COCY species' host trees along approximately 2.8 km of the Ashton and upper Enkiang trails; not all nest trees were found. Material was identified to genus, and occasionally to species, at the Brunei Herbarium. Representation of plant families and genera among sampled hosts was compared to that summed from three tree plots maintained by KBFSC and located along the same trails.

Statistical Analyses

All analyses were done in JMP version 4.0.4 (SAS Institute 2001). Conservatively, where multiple microbes were present but inseparable without cloning (one case each for fungi in COCY fiber and carton samples), focal nest associates were considered to be absent.

RESULTS

Mandibular Gland Chemistry

Augmenting published data, Table 1 summarizes the volatile chemistry of mandibular gland products for the eight COCY species colonizing artificial nests. Pending comparisons of collections with type specimens, just one (*Camponotus* [*Colobopsis*] *saundersi*) has been identified to species,

Table 1. By species, percentage representations of compounds (including fatty acid methyl esters) in mandibular gland (MG) products; t = trace. See text and Jones et al. (2004) for details. Data are listed by voucher numbers (DWD KB collection series) and species acronyms (from Cook 2008). Product colors are identified below: w = white; y = yellow; o = orange; r = red (occasionally pink or peach).

	Species							
MG product	05B-50 'LE'* (r)	02-118 'RHYG' (y)	02-108 'YG' (y)	07B-T2 'ICY' (w)	11-Feb 'CL' (w)	Feb-64 'AR' (w)	02-21 'SA' (w)	05A-37 'nrSA'[1] (w)
Phenolics								
m-Cresol (**1**)					14		1	37.5
Resorcinol (**2**)	10							
6-Methylsalicylic acid (**3**)[2]					30			7.5
2,4-Dihydroxy-acetophenone (**4**)	25		1		3	4	75	3.2
2,4,6-Trihydroxy-acetophenone (**5**)	1.8		2			15		
2-Methyl-5,7-dihydroxy-chromone (**6**)		24	21			1		
Orcinol (**8**)						t		
Terpenoids								
Citronellal								
Citronellol								
Citronellic acid								t
Isopulegol								
(6R)-E-2, 6-Dimethyl-2-octen-1,8-dioic acid (**9**)						44		16.5
E-8-Hydroxy-3, 7-dimethyl-6-octenoic acid (**10**)						t		

[1] Means of two analyses for same species.
[2] In insects, compound **3** can be an intermediate in the production of **1** (Birch and Donovan 1953).

and most COCY species are unidentified or undescribed. The remaining collections are referenced by descriptive acronyms and voucher numbers (see Acknowledgements). Compound numbers correspond to those in Jones et al. (2004); we omit previously reported aliphatics occurring just in ant gasters, and therefore not MG products (authors' unpublished data). One or more of several phenolic acetogenins (compounds **1–6**) and terpene diacids (**9–10**) occur in each colonizing species. Corrosively irritant m-cresol (**1**) is a major component in several derived species (Table 1 vs. Cook 2008). None of the sampled COCY species failing to colonize artificial nests possesses significant quantities of m-cresol in MG products (Jones et al. 2004 and T.H. Jones, unpublished data), nor do those taxa maintain satellite nests in fallen wood (Cook 2008 and authors' unpublished data).

Three of the polyacetate-derived aromatics (compounds **4–6** in Table 1 below), at least one of which occurs in each species, determine the bright colors of MG products (Table 1, Jones et al. 2004). Independently of which product predominates, these colors are pH-dependent in the range of 5.6 (white) to 7.8 (pink or red), and cream-to-yellow or orange at intermediate pH (Jones et al. 2004).

Nest pH and Occupation

In preliminary trials, and contrary to expectation, nest wall pH was invariant (≈ 4) over all early sampled nests with and without ants, as well as in natural nests in preliminary trials. Among ant-occupied nests were those used by COCY species with MG products ranging from white ('CL' and 'SA') to yellow ('YG'), and red ('LE'), one nest each for *Camponotus* species 06B-04 and *Tetramorium* sp. 06B-05 (a myrmicine ant). Based on lack of variation in pH, we eventually discontinued measurements.

Within 18 months (usually sooner), COCY species had colonized 44% of the

25 nests located adjacent to nests of known colonies, and 48% of those not colonized by other species (*Polyrhachis* and *Tetramorium*). Six of eleven uncolonized nests were located near natural nests of taxa not known to inhabit natural fallen wood (see Cook 2008), and several others had been breached by water. Of 30 artificial nests placed 5–8 m from natural nests in a major foraging direction, COCY species occupied 33.3%, or 34.5% of nests not occupied by other taxa. Eleven of the uncolonized nests in this set were stationed near species not known to nest naturally in dead wood, and some others had been breached by water or disturbed by sandalwood poachers. Both 'LE' and 'YG' moved into artificial nests (the latter species with brood), despite apparently not nesting naturally in fallen wood. Not all COCY-occupied nests were sampled for microbes, because some had been colonized by just small colony fragments.

Microbes in Nest Wall Fiber

Six COCY species colonized artificial nests and/or were harvested from natural nests, and microbes from these two nest types were lumped in subsequent analyses. For 24 fiber samples from COCY nests, replicate PCRs failed to yield 'hits' in ten cases with universal bacterial primers but in just three cases with universal fungal primers. Comparable data for 17 unoccupied nests were seven and three, respectively, and for carton samples, were seven and zero of 24 samples. The acidic environment of nest cavity walls may generally favor fungi over bacteria, but we cannot rule out influences of culture conditions specific this study, or of differentially 'successful' PCRs.

In Table 2, microbial data are presented with COCY species organized by nesting habits. Workers of all fallen wood nesters commute extensively on the ground, in contact with soil microbes. So far as is known, both 'YG' and 'RHYG' nest only in live trees, but the two taxa differ in exposure to soil microbes. 'YG' has extensive contact with leaf litter and soil, whereas observations of four different colonies reveal 'RHYG' commuting along dead and live stems, rather than over leaf litter or soil. Both nesting and foraging in the canopy, 'LE' has the least exposure to soil microbes, except as those organisms inhabit decaying leaf litter in the canopy itself.

For the most common fungal and bacterial taxa, Table 2 reports microbial data by colony, nest sample (including multiple nests per colony), and total nest samples over time (including replicate samples from individual artificial nests). Representatives of one bacterial genus and one fungal genus appeared repeatedly in both nest fiber and carton from COCY species' nests. These were, respectively, *Burkholderia* and *Trichoderma* (anamorph = asexual form)/*Hypocrea* (teleomorph = sexual form). GenBank accession numbers are in three sets: GQ306157-GQ306183 (file Anderson_*Burkholderia*.sqn), GQ332537 (file Anderson_*Burkholderia*.sqn), and GQ306184-GQ306202 (file Anderson_*Hypocrea*.sqn).

Only microbes from fiber can be compared with those from unoccupied nests, which lacked carton. At the colony level, over-representation in association with COCY is clearest for *Trichoderma* species, which were detected in nests of 50% of 14 COCY colonies and 70% of ten colonies from taxa nesting regularly in dead wood. (In nest carton, they were found in 69% of 13 colonies and 70% of ten colonies nesting in dead wood.) In contrast, members of this genus were detected in none of 17 unoccupied nests, and in just one of seven nests inhabited by other ant taxa. Presence of *Trichoderma* differed significantly across the three nest types in nominal logistic regression ($\chi^2_{[2]} = 13.96$, $P < 0.0009$, $N = 38$). Nevertheless, it could be argued that nests of COCY species were sampled more thoroughly than were other nests, due to our sometimes having sampled fiber from multiple nests per colony, and/or individ-

Table 2. Occurrences of the most common bacterial and fungal genera detected in artificial nests at KBFSC. Sample sizes are in parentheses for N = numbers of colonies (some sampled by multiple nests, and some nests sampled repeatedly), numbers of nests (independent nests, but sometimes more than one per colony), and total nest samples (including repeat samples of nests restored and returned to the field).

Species	+ *Burkholderia* spp.[1]			+ *Trichoderma* spp.[2]		
	Colonies sampled (N)	Nests sampled (N)	Total nests over time (N)	Colonies sampled (N)	Nests sampled (N)	Total nests over time (N)
NEST WALL FIBER						
COCY spp. Nests						
Satellite nests in dead wood						
'nrSA'	1 (1)	1 (1)	1 (1)	1 (1)	1(1)	1 (1)
'SA'[5]	3 (4)	3 (6)	3 (6)	1 (4)	1 (6)	1 (6)
'AR'	1 (2)	1 (3)	1 (4)	2 (2)	2 (3)	3 (4)
'CL'	2 (2)	2 (2)	2 (2)	2 (2)	2 (2)	2 (2)
'ICY'[4]	1 (1)	2 (4)	2 (4)	1 (1)	1 (4)	1 (4)
No satellite nests in dead wood						
'RHYG'[4]	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (0)
'YG'	1 (2)	1 (3)	1 (4)	0 (2)	0 (3)	0 (4)
'LE'	0 (1)	0 (1)	0 (2)	0 (1)	0 (1)	0 (2)
Unoccupied nests						
All	5 (17)	5 (17)	5 (17)	0 (17)	0 (17)	0 (17)
Nests occupied by non-COCY spp.						
Tetramorium sp. KB06B-05	1 (1)	1 (1)	1 (1)	0 (1)	0 (1)	0 (1)
Camponotus sp. 1 KB06B-04	1 (3)	1 (3)	1 (3)	0 (3)	0 (3)	0 (3)
Polyrhachis [*Polyrachis*] sp. KB07A - 02	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
Polyrhachis sp. 1	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
Polyrhachis sp. 2 (*hector* group) KB07A-03	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
CARTON NEST SHELVING						
COCY spp. Nests						
Satellite nests in dead wood						
'nrSA'	1 (1)	1 (1)	2 (2)	1 (1)	1 (1)	1 (2)
'SA'	4 (4)	5 (6)	5 (7)	1 (4)	1 (6)	1 (7)
'AR'	1 (2)	1 (3)	1 (4)	2 (2)	2 (3)	2 (4)
'CL'	1 (2)	1 (2)	1 (2)	2 (2)	2 (2)	2 (2)
'ICY'[4]	0 (1)	0 (2)	0 (2)	1 (1)	2 (2)	2 (2)
No satellite nests in dead wood						
'RHYG'[5]	0 (1)	0 (1)	0 (1)	1 (1)	1 (1)	1 (1)
'YG'[5]	0 (1)	0 (2)	0 (5)	1 (1)	1 (2)	1 (5)
'LE'	0 (1)	0 (1)	0 (2)	0 (1)	0 (1)	0 (2)
Non-COCY spp.						
Camponotus sp. 1[3]	1 (3)	1 (3)	1 (3)	0 (3)[3]	0 (3)[3]	0 (3)[3]

[1] BLAST hits included *Burkholderia* spp.: *tropica* (most common, 'SA'), *ginsengisoli* (in GenBank as *koreensis*, not a legitimate name, 'SA'), *phenazinium* (*Polyrhachis* sp. 1), and *terricola* (CL); hits for a nest occupied by *Camponotus* sp. 1 were near *uname* and *nodosa*.

[2] BLAST hits included *Trichoderma* spp. or *T. viride* (anamorph) and *Hypocrea lutea* (identifications to be checked by I. Druzhinina).

[3] In one sample of SA fiber and one sample of *Camponotus* sp. 1 carton, multiple fungal taxa not discriminated without cloning; *Trichoderma* cannot be ruled out.

[4] Three of four sampled ICY nests were natural and in fallen wood on the forest floor; one natural nest possessed both *Burkholderia* and *Trichoderma*, and *Burkholderia* alone was found in a second natural nest. The sole sampled 'RHYG' nest was in a recently dead, standing host.

ual nests repeatedly over time. To circumvent this problem, we repeated our analysis including data from just the first of replicate samples of individual nests, and also classifying *Trichoderma* as absent from half the colonies where it was found in just half of all replicate nests per colony. Even in this more conservative analysis, *Trichoderma* was over-represented in COCY nests (35.7% of samples) relative to the other two nest types from which it was either absent or rare ($\chi^2_{[2]} = 9.16$, $P = 0.01$, $N = 38$).

Among COCY taxa, *Trichoderma* was detected exclusively and significantly more often in species nesting regularly in fallen wood on the forest floor (Table 2; $\chi^2_{[1]} = 7.19$, $P = 0.007$, $N = 14$). All but one of those taxa ('AR') produce m-cresol as a component of MG product; as a fraction of total volatiles, m-cresol is least well represented in 'SA' (Table 1), and detection of *Trichoderma* was also least consistent in nests of that species.

Burkholderia bacteria were detected in 64.3% of 14 COCY spp. nests but were also found in smaller percentages of both unoccupied nests (29.4%) and nests housing other ant taxa (42.9%). At the colony level, the bacteria were not statistically over-represented in COCY nest fiber in the less conservative analysis ($\chi^2_{[2]} = 3.85$, $P = 0.15$, $N = 38$). However, *Burkholderia* were detected marginally more frequently in (lumped) ant-occupied nests than in vacant nests ($\chi^2_{[1]} = 2.98$, $P = 0.08$), and they were significantly more common in COCY nests than in unoccupied nests ($\chi^2_{[1]} = 3.84$, $P = 0.05$, $N = 31$). When each of these three analyses was repeated with a more conservative data set (modeled on that for *Trichoderma* above), none was statistically significant ($P \gg 0.05$ in each case). Finally, lumping COCY species in each category, *Burkholderia* was more common in nest fiber of taxa regularly inhabiting fallen wood, than in other taxa ($\chi^2_{[1]} = 3.74$, $P = 0.05$, $N = 14$).

In nest fiber, *Burkholderia* and *Trichoderma* co-occurred significantly more often at the level of ant colony than predicted by chance ($\chi^2_{[1]} = 3.94$, $P < 0.05$, $N = 13$, in nominal logistic fit with the equivocal nest with multiple fungi omitted). Based on their separate rates of detection in COCY colonies, independence would have predicted co-occurrence in 41.3% (64.3% × 64.3%) of nests. However, *Trichoderma* was present in 85.7% of nests in which *Burkholderia* was confirmed, and *Burkholderia* in 75% of nests where *Trichoderma* was detected. After removing the four colonies of taxa nesting naturally only in live wood, the relationship is no longer significant ($P \gg 0.05$, suggesting that it depends largely on differences in species' nesting habits. Identical comparisons for carton samples revealed no evidence of association between the two categories of microbes, mainly because *Trichoderma* was absent altogether from 'SA' (little m-cresol) carton, despite presence of *Burkholderia* in almost all such samples (Table 2).

Endophytic Fungi of Leaves and Roots

Endophytic *Trichoderma* were documented in two of three leaves and one of three roots sampled. One of the two positive determinations for leaves was for a canopy host and resource plant of 'LE', and the other was from an understory resource sapling of 'SA'. *Trichoderma* was not detected in an understory palm utilized heavily by 'YG'. *Burkholderia* were detected in none of three leaves, but in one of three roots, sampled; that single root was from an understory resource plant of 'BBQ', the sister species of 'LE' (Cook 2008), but a species commuting regularly on the ground. *Trichoderma* was detected in that same root.

Ant Associations with Host Trees

Both individually and as a group, COCY species nested in a diversity of live host species (Table 3). Several host taxa possessed extrafloral nectaries (EFNs), producing food rewards for ants on leaves or reproductive structures (*Ixora* fruits), but

most host species did not. Across all ant taxa, and despite lack of species-specificity in ant-host associations, certain taxa lacking EFNs were overrepresented as hosts relative to their family and genus level abundances in forest plots. Accounting for ~ 21% and ~34% of 56 identified hosts, respectively, both Fabaceae and Phyllanthaceae were statistically overrepresented relative to 'other plant families' from which hosts had been identified (Likelihood Ratio [LR] $X^2_{2, 2458} = 8.08$, $P = 0.0030$ for Fabaceae, and LR $X^2_{2, 2458} = 13.87$, $P = 0.0002$ for Phyllanthaceae). Two thirds of hosts in the Fabaceae were species of *Fordia*, but because *Fordia* also comprised 70% of all family members in tree plots, it was actually significantly under-represented as a COCY host tree (LR $X^2_{1, 215} = 8.01$, P = 0.0047). *Antidesma* and *Cleistanthus* were approximately equally represented as hosts and together accounted for 89.5% of the Phyllanthaceae. Each was significantly over-represented as hosts compared to the distribution of abundances as a whole (focal genus versus lumped other genera: LR $X^2_{1, 99} = 36.47$, $P < 0.0001$ for *Antidesma*, and LR $X^2_{1, 215} = 14.16$, P = 0.0002, for *Cleistanthus*).

DISCUSSION

Frequent colonization of artificial nests (~38% overall) suggests that suitable living space is limiting for polydomous COCY species and other cavity-nesters at KBFSC, perhaps especially so for taxa in which workers lack metapleural glands (all but *Tetramorium* in Table 2). Uniformly low pH of nest cavity walls is likely determined by brown-rot wood decay fungi (e.g. Humar et al. 2001) and, consistent with our data, may generally favor fungi over bacteria (e.g. Bot et al. 2002). Experimental data also reveal over-representation of *Trichoderma* fungi in artificial nests colonized by COCY taxa regularly maintaining satellite nests in fallen wood; four of these five species are the only taxa possessing m-cresol as a MG product. Although *Burkholderia* bacteria were detected most frequently in COCY species' nests, they were overrepresented there only in relation to unoccupied nests, and only in the least conservative analysis. Below, we review these results in the context of a phylogeny of COCY species (Cook 2008), ant habits, and known attributes of *Trichoderma* and *Burkholderia*.

Nest Site Limitation

Overall, evidence for limitation of nest space is consistent with phylogenetic data suggesting that shortages of cavity space could have driven progressively greater use of understory nests and, eventually, nests in fallen wood, as the increasingly derived character states (Cook 2008). Because nests were placed adjacent to known colonies, high rates of colonization by COCY species are due partly to high discovery rates, but they also indicate that suitable nesting space was limiting for many of these colonies. Colonization rates might have been higher still, had we sampled just taxa known to nest in the understory. For other species, artificial nests positioned at bases of host and resource trees may have been unacceptable despite substantial worker traffic to the understory and on the ground. Additionally, in 'RHYG', which nests throughout trunks of small trees, nesting space may not be limiting until death of the host tree, which it regularly kills (authors' unpublished data). Finally, both 'YG' and 'LE' adopted artificial nests despite not nesting naturally in fallen wood.

To the extent that ants procure food from their hosts, limited nest space may correlate with food limitation. A minority of hosts provide extrafloral nectar (Table 3), and frequent mortality of 'RHYG' host trees suggests that this species drains resources from live hosts, despite not tending trophobionts inside. Trunks are gradually hollowed out, and bark is stripped from external surfaces where workers harvest cambial heteroplasias at sites of injury. Similar behavior is reported

Table 3. Ant-host associations: entries are numbers of occurrences. Not all hosts were located. Asterisks mark taxa with extrafloral nectaries.

Ant sp.	'SCY'	'nrSA'	'SA'	'AR'	'CL'	'RHOG'	'LCY'	'YG'	'RHYG'	'BBQ'	Not recorded
Plant Family and Genus											
Anacardiaceae											
Gluta							1				
Celastraceae											
Lophopetalum									1		
Dipterocarpaceae											
*Shorea**			1								
Euphorbiaceae											
*Blumeodendron**							1				
*Macaranga (hullettii)**			1								
*Mallotus**			2		1						
Fabaceae											
*Albizia**									1		
*Archidendron**			1							1	
Fordia			1	3	3				1		
Callerya										1	
Meliaceae											
Aglaia											1
Myristicacae											
Horsefieldia				1							
Myrtaceae											
Memecylon			1								
Syzigium								1			
Oleaceae											
*Anacolosa**						1					
Phyllanthaceae											
Antidesma			5						3	1	
Aporosa					1						
Cleistanthus	1		4				1		2		
Drypetes									1		
Polygalaceae											
Xanthophyllum					1		1				
Rubiaceae											
*Ixora**		1	1								
Praravinia					1						1
Sapindaceae											
*Pommetia**								1			
Simaroubaceae											
Eurycoma			2								
Tiliaceae											
Microcos				1							
Violaceae											
Rinorea				1							

* Hosts with EFNs

for *Camponotus* [*Colobopsis*] *quadriceps* (Davidson and McKey 1993), a phytoecious resident of *Endospermum* (Emery 1925), and a New Guinea relative of COCY species. In other species, very high worker activity can occur throughout the day at some dead wood nests lacking brood ('CL'). Finally, space could be associated with food in the form of nest shelving. In lab-housed colony fragments, workers of various COCY species deposited liquid from sugar-soaked cotton balls on carton fragments reserved from nests and supplied to the ants. Initially dry and fibrous, the carton even-

tually turned dark black, whereupon workers harvested the loose black material, leaving dry fibrous carton of diminished thickness.

Ant-microbe Associations

Remarkably, even light sampling of cultured microbes from few colonized nests revealed representatives of one bacterial genus (*Burkholderia*) and one fungal genus (*Trichoderma*) as frequent nest associates of COCY species. *Trichoderma* species, filamentous Ascomycota (Hypocreales, Hypocreaceae) were cultured mainly from COCY species' nests. Because nests were assigned haphazardly to field locations, this result cannot be explained by preexisting endophytic infections of lumber used in nest construction. Presence of *Trichoderma* and *Burkholderia* in a nest occupied by *Polyrhachis* (informal subgenus *Polyrhachis*) workers is noteworthy, given that members of this group share and defend territories with COCY species (Davidson et al. 2007). Although these species nest in soil, where they line cavity walls with wood fiber (changed out periodically), they can maintain 'pavilions' without brood in standing dead wood (authors' unpublished observations).

Across COCY species, *Trichoderma* was detected in nest fiber of just the five taxa regularly maintaining satellite nests in fallen wood (Table 2), and four of these taxa produce *m*-cresol as a component of MG product (Table 1). The exception is 'AR', where the major component is a diterpene (**9**). Comparing the four taxa with *m*-cresol, frequency of *Trichoderma* was lowest in 'SA', coincidentally the species with the lowest concentration of this compound (1–5% across repeat samples, versus 30–98%, respectively, Table 1, and authors' unpublished data). In carton, *Trichoderma* was detected at least once in all COCY taxa except 'SA' and 'LE', the canopy-restricted species. Together, these data suggest that *m*-cresol may favor *Trichoderma* over other fungi (see below). Although certain categories of diterpene acids also exhibit strong antifungal activity (Kopper et al. 2005), the response of *Trichoderma* to such compounds remains unexplored.

To account for distributions of *Trichoderma* and *Burkholderia* across artificial nests, we propose the following hypothetical scenario, consistent with our data and ancillary observations. Widespread in soils (Coenye and Vandamme 2003), or in soils and decaying wood (e.g. Kubicek and Harman 1998), members of both genera may first have colonized artificial nests via passive dispersal on tarsi of ant taxa commuting regularly over the ground. (COCY species do not actually forage in leaf litter.) The same microbes may not have been picked up in abundance by species with activities confined to vegetation, nor might *Trichoderma* have thrived in nests of terrestrially commuting taxa lacking sufficient *m*-cresol to convey a competitive advantage to these fungi (which degrade the compound; e.g. Bruce and Highly 1991 and Atagana et al. 2002; Karetnikova and Zhirkova 2005) over other fungi. (Evidence also indicates that *Burkholderia* can use *m*-cresol and other aromatics as carbon sources [e.g. Shields et al. 1995; Caballero-Mellado et al. 2007]). MG products could have arrived at nest walls via their volatility or been applied as nest wall fiber was stripped and macerated into carton shelving. Similar 'arrival and survival' characteristics of *Burkholderia* and *Trichoderma* have could have contributed to their positive association in nest fiber.

Clearly, our simple approach to microbial sampling could have missed other common associates of the ants, e.g. taxa concentrated in other parts of the nest, or more readily cultured on other media. (However, samples plated onto methanol and acetate agars during one sampling period typically yielded the same organisms as did cultures on MEA). Additionally, plate cultures are not unbiased methods for environmental sampling but favor taxa

requiring high rates of resource supply (see below), which central-place foraging ants may nevertheless provide.

Potential Benefits of Association with Microbes

Both *Burkholderia* and *Trichoderma* have also been isolated from nests of leaf-cutter ants (Attinae), with the former genus reported to have antibiotic activity against entomopathogenic fungi (Santos et al. 2004), and the latter to be commensals or mild parasites of the fungus garden (Currie et al. 1999a; Bot et al. 2002; Rodrigues et al. 2008). Although as mycoparasites, *Trichoderma* may damage attine fungal gardens, they are beneficial in human agriculture where they are exploited commercially as biological control agents (e.g. Kubicek and Harman 1998) that arrest growth of wood decay fungi and plant pathogens. We speculate that *Trichoderma* could play a similar role in nest hygiene by protecting COCY workers and brood from entomopathogenic fungi and bacteria. A subset of the mechanisms by which *Trichoderma* fungi control plant pathogens (Kredics et al. 2003; Howell 2003) could directly harm nest pathogens. If nests in live hosts also contain these fungi, other mechanisms could potentially modify anti-pathogen defenses of live host trees to the ants' advantage. Modes of action include: (a) endogenous and exogenous production of chitinolytic enzymes that degrade the polysaccharides, chitin and β-glucans conveying rigidity and integrity to fungal cell walls; (b) production of proteases that inactivate hydrolytic enzymes of pathogenic fungi, breaking them down to peptides and amino acids so that fungi cannot invade host tissues; (c) competitive replacement of fungal pathogens within plant tissues; (d) production of antibiotics; (e) synergistic actions of chitinases with both antibiotics and proteases; (f) metabolism of spore germination stimulants; (g) induction of plant-produced terpenoid defenses, potentially fungitoxic peroxidases, and pathogenesis-related proteins in roots and leaves of live plants themselves, possibly including COCY live host trees. Plant-produced chitinases and proteases should have activities similar to those of comparable *Trichoderma* enzymes. Just the presence of *Trichoderma* in the rhizosphere, contacting but not invading plant tissues, can induce systemic plant defenses that are potentially effective against bacterial as well as fungal pathogens (Harman et al. 2004). Both mycelial growth and enzyme production by *Trichoderma* increase in mesic environments and acidic substrates (Kredics et al. 2003), common conditions in fallen wood on the rainforest floor.

COCY species lack pupal silk, which may protect other ant taxa (e.g. *Polyrhachis* spp.) from nest pathogens and may be especially effective where microbial antagonists of such pathogens are interwoven with silk (e.g. Kaltenpoth 2007, for wasps). With naked pupae, COCY colonies and species not associating with *Trichoderma* must maintain nest hygiene by other means. Laboratory-housed workers of 'YG' colony fragments uniquely deposited abundant MG product on floors and walls of plastic nest chambers, and those of 'LE' lined nest chambers with cotton shredded from *Candida* yeast-occupied sugar-soaked cotton balls offered as food. Both behaviors could be related to nest hygiene. Additionally, from 'SA' nests, we isolated *Verticillium insectorum*, a fungus reported to be parasitic on *Trichia* slime molds (Rogerson and Stephenson 1993), which our primers would not have detected. Like *Trichoderma*, slime molds consume microbes in decaying vegetation.

We cannot presently rule out direct or indirect positive effects of *Trichoderma* infections on food production inside nests and on foraging substrates, or on expansion of nest space. Whether chitinolytic enzymes and proteases are produced by *Trichoderma*, and/or elicited in live host and resource plants, such enzymes might increase resource availability for leaf-graz-

ing ants feeding on products of fungal breakdown. *Trichoderma* propagules could also be among spores digested *in situ* in a worker's infrabuccal cavity to form lipid-rich products (authors' unpublished data, see also Hansen et al. 1999, for *Camponotus modoc*). Further, by stimulating sporulation of some fungi (Brazier 1971), *Trichoderma* might increase spore availability to ants as food. Finally, consistent with saprophytic life styles, *Trichoderma* species possess rich arsenals of extracellular enzymes involved in degradation of cellulose (endo- and exo-glucanases, β-glucosidase, cellobiohydrolase), lignin and hemicellulose in plant cell walls (laccase, peroxidase, xylanase, xylosidase, pectinase and pectin lyase), starch (α-amylase), and chitin (chitobiosidase, N-Acetyl-β-D-glucosaminidase) (e.g. Harman and Kubicek 1998; Kredics et al. 2003). These enzymes could potentially make sugars available to stem-mining or leaf-grazing COCY taxa, just as activities of comparable enzymes subsidize growth of fungal associates of leaf-cutter ants (Gomes De Siqueira et al. 1998; Schiøtt et al. 2008), and cavity space could in the process. Whether *Trichoderma* retard wood decay by mycoparasitism (Shigo 1989; Bruce and Highly 1991; Bruce et al. 2000) or hasten it via cellulolytic activities may depend on species and strain.

Burkholderia bacteria may grow especially well in association with ant waste and perhaps benefit ants through waste recycling. All identified species (footnote in Table 2) belong to the *Burkholderia* clade containing all but one of the plant-associated diazotrophic species, including root-nodulating members in which presence of the nifH gene has been confirmed (Coeyne and Vandamme 2003; Reis et al. 2004; Martínez-Aguilar et al. 2008). Various species in this group produce ureases and grow well aerobically on ammonium substrate (Caballero-Mellado et al. 2004; Reis et al. 2004). If *Burkholderia* recycle COCY species' waste (see also Van Borm et al. 2002, for congeners inhabiting gut pouches of a common KBFSC pseudomyrmecine), this might explain how nest cavity walls remain remarkably free of fecal contamination in nests tightly packed with workers and brood (authors' observations). Where studied, N-fixation by diazotrophic *Burkholderia* occurs under microaerobic conditions (Reis et al. 2004) such as could exist at night in very crowded nests of strictly diurnal COCY species. Both temperature and pH levels in ant nests of the equatorial KBFSC rain forest are in the range in which N-fixation by these species proceeds well (25–37°C, optimum 30°C; pH 4.5–6.5; see Reis et al. 2004).

If *Burkholderia* were to enhance N availability in the nest, this process could potentially contribute to nest longevity. In high lignin substrates like wood, N fertilization favors breakdown of relatively easily decomposed cellulose by a variety of decomposers and leaves behind more recalcitrant lignocellulose (Fog 1988), which fewer microbes can degrade. Time and again, we noted that COCY nest cavity walls in natural fallen wood were remarkably hard and extremely difficult to crack open, despite extensive decay of external wood. Hardening of cavity walls should lengthen the useful life spans of nests in dead wood.

Endophytic *Burkholderia* and *Trichoderma*

Some *Trichoderma* and *Burkholderia* also form sustaining endophytic infections in roots, stems, and leaves (e.g. Bailey et al. 2006; Coenye and Vandamme 2003; Compant et al. 2005a,b). Both taxa typically contact plants in the rhizosphere, where they stimulate upregulation of systemic plant defenses, spread into roots (van Loon et al. 1998; Harman et al. 2004; Compant et al. 2005b; see also e.g. Carroll 1988; Arnold 2003), and can enhance nutrient capture (Caballero-Mellado et al. 2007 for *Burkholderia*). Bacteria disperse to stems and leaves through xylem (Compant et al. 2005b), but many fungal endophytes sporulate in leaf litter and colonize new growth via spore

dispersal by wind and/or insect vectors. Endophytes can potentially protect hosts from pathogen damage (e.g. Redman et al. 2001; Arnold et al. 2003), and this outcome is the motivation for commercial use of mycoparasitic *Trichoderma* in agricultural systems.

Several factors suggest that relationships between COCY spp. and endophytes warrant further study. First, and remarkably given the extraordinary diversity and patchiness of endophytes in tropical vegetation (e.g. Arnold and Lutzoni 2007), our highly inadequate sampling of plant material detected endophytic *Trichoderma* at 'leaf stops' in two of three leaves where ants foraged, and in one of three roots of sampled host/resource plants (none were over-represented host taxa). We could easily have missed both organisms where present, e.g. by failing to target plant parts from which the bacteria have been reported (Compant et al. 2005b). Second, all COCY species forage by 'grazing' adaxial leaves for microscopic rewards (Davidson et al. 2004) that, in addition to epiphylls, could include products mediated by foliar endophytes. Third, COCY species would seem to be ideal spore dispersal agents. In many such species, hundreds-to-thousands of workers commute regularly over the ground between central and satellite nests, and between all nests and both resource plants and pathways into the canopy. Fungal spores are common in buccal pellets, and in more derived species, pellets may be deposited on leaves via a peculiar 'shuddering' behavior (Cook 2008).

Finally, although COCY species do not exhibit species-specificity to host trees, certain plant taxa are over-represented as hosts for the ant clade as a whole, and those taxa do not produce ant rewards (Table 3). If the ants are capable of manipulating microbes in the nest, they may also have evolved to recognize and respond to host endophytes with potential to influence colony success. The fact that both over-represented hosts are members of the Phyllanthaceae (formerly included in Euphorbiaceae, see Wurdack et al. 2004) is interesting in light of findings that the capacity of plant pathogens to infect different host taxa varies inversely with phylogenetic distance (Gilbert and Webb 2007), and that beneficial endophytes may be closely related to pathogens (e.g. Schulz et al. 1999; Wang et al. 2009). Finally, *Cleistanthus* hosts were regularly infected by *Fusarium* (like *Trichoderma* a Class 2 endophyte; Rodriguez et al. 2008) (Hairunizam Hj. Panjang, Plant Pathology Unit, Brunei Agriculture Research Centre, pers. comm.), certain species of which convey resistance to fungal pathogens (Schulz et al. 1999). If that is happening here, *Fusarium*-infected hosts may also afford protection against nest pathogens.

Coda

In the context of ant associations with microbes, it seems possible that the dramatic defense of foraging territory by suicidally exploding ants could have arisen in part as a means of preventing contamination by alien fungal strains or species. As a first step in evaluating this possibility, work is under way to determine the degree to which relationships between ants and *Trichoderma* might be species- or strain-specific. If they are, fungi of different ant species could be competitors of one another, with fungal chemistry possibly mediating ant recognition of alien fungi and eliciting ant behaviors that guard against contamination (e.g. Bot et al. 2001b; Zhang et al. 2007, for attine ants and their fungi). Competition between fungal species or strains might be costly to plants and ants as well as fungi.

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